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Regulation of the Epithelial Sodium Channel by Serine Proteases in Human Airways*

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Scott H. Donaldson‡§¶, Andrew Hirsh‡, Dong Chen Li‡, Ginger Holloway‡, Julie Chao‖, Richard C. Boucher‡§, and Sherif E. Gabriel‡**

*From the ‡Cystic Fibrosis Research and Treatment Center, §Department of Medicine, and **Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599 and the ‖Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425*

The epithelial sodium channel (ENaC) constitutes the rate-limiting step for sodium absorption across airway epithelia, which in turn regulates airway surface liquid (ASL) volume and the efficiency of mucociliary clearance. This role in ASL volume regulation suggests that ENaC activity is influenced by local factors rather than systemic signals indicative of total body volume homeostasis. Based on reports that ENaC may be regulated by extracellular serine protease activity in *Xenopus* and mouse renal epithelia, we sought to identify proteases that serve similar functions in human airway epithelia. Homology screening of a human airway epithelial cDNA library identified two trypsin-like serine proteases (prostasin and TMPRSS2) that, as revealed by *in situ* hybridization, are expressed in airway epithelia. Functional studies in the *Xenopus* oocyte expression system demonstrated that prostasin increased ENaC currents 60–80%, whereas TMPRSS2 markedly decreased ENaC currents and protein levels. Studies of primary nasal epithelial cultures in Ussing chambers revealed that inhibition of endogenous serine protease activity with aprotinin markedly decreased ENaC-mediated currents and sensitized the epithelia to subsequent channel activation by exogenous trypsin. These data, therefore, suggest that protease-mediated regulation of sodium absorption is a function of human airway epithelia, and prostasin is a likely candidate for this activity.

The amiloride-sensitive epithelial sodium channel (ENaC)[1] (1) constitutes the rate-limiting step of sodium transport across many epithelia, including the kidney, colon, and lung (2). In the kidney, the regulation of ENaC activity is necessary for the proper maintenance of total body extracellular volume and blood pressure and is responsive to systemic hormonal signals, such as aldosterone and vasopressin. In the lung, regulated ENaC activity is essential for the maintenance of airway surface liquid (ASL) volume/depth. The physiologic importance of the relationship between ENaC activity in airways and ASL volume is highlighted by two disease states, cystic fibrosis (CF) and pseudohypoaldosteronism type 1. In CF, the loss of functional cystic fibrosis transmembrane conductance regulator (CFTR) protein results in excessive ENaC activity (3, 4), sodium hyperabsorption (5), and ASL volume depletion (6). The depletion of ASL volume, in turn, causes the overlying mucus gel to collapse onto cilia and the cell surface, thus disrupting mucociliary clearance (7). In contrast to CF, pseudohypoaldosteronism type 1 patients have mutations in genes encoding ENaC subunits, which result in diminished sodium absorption, increased ASL volume, and markedly accelerated rates of mucociliary clearance (8). CF and pseudohypoaldosteronism type 1, therefore, provide striking evidence that regulated ENaC activity is a prime determinant of ASL volume and mucociliary function.

The regulation of ENaC has proven to be tissue-specific, and many previously characterized mechanisms pertinent to ENaC activity in the kidney may not be relevant in airway epithelia. For example, dietary salt intake and circulating mineralocorticoid levels modulate ENaC activity in the kidney but do not affect ENaC function in airways (9). Similarly, while signals that raise intracellular cAMP in the kidney (*e.g.* vasopressin) increase ENaC currents by stimulating channel insertion into the plasma membrane (10, 11), this second messenger pathway has little effect on amiloride-sensitive currents in normal airways (4). Finally, while patients with Liddle's syndrome manifest renal sodium hyperabsorption due to ENaC mutations (12–14), they do not appear to have sodium hyperabsorption across airway epithelia (15). Based upon these differences, we speculate that 1) ENaC regulation is tissue-specific and 2) local regulators responding to stimuli in the immediate channel vicinity, rather than systemic signals, are likely to play a dominant role in the lung.

Vallet *et al.* (16) identified one such candidate for the local regulation of ENaC in 1997. These investigators cloned a trypsin family serine protease, xCAP1 (channel-activating protease), from a *Xenopus* kidney epithelial cell line (A6) using a functional complementation assay designed to detect activators of ENaC in the *Xenopus* oocyte expression system. In this assay, a 2–3-fold activation of ENaC was observed when xCAP1 was coexpressed with rat or *Xenopus* ENaC in oocytes. This effect appeared to rely on extracellular xCAP1 protease activity, since incubation in media containing a serine protease inhibitor (aprotinin) prevented ENaC activation. Northern analyses identified message for xCAP1 in *Xenopus* tissues where ENaC was also expressed, including the kidney, gut, lung, and skin. They further showed that endogenous serine protease activity regulated basal ENaC currents in the *Xenopus* A6 renal epithelial cell line. As in studies of xCAP1 in

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¶ To whom correspondence should be addressed: Cystic Fibrosis Research and Treatment Center, 6019 Thurston Bowles Bldg., CB# 7248, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-966-9198; Fax: 919-966-7524; E-mail: Scott_Donaldson@med.unc.edu.

[1] The abbreviations used are: ENaC, epithelial sodium channel; ASL, airway surface liquid; CF, cystic fibrosis; RT, reverse transcriptase; CFTR, cystic fibrosis transmembrane conductance regulator.

oocytes, inhibition of extracellular serine protease activity with aprotinin significantly diminished ENaC currents, whereas the subsequent addition of exogenous trypsin rapidly increased amiloride-sensitive sodium transport. The first mammalian homologue of xCAP1 was identified in a mouse kidney cell line ($mpkCCD_{c14}$) by RT-PCR with degenerate primers (17). The mouse homologue, mCAP1, increased amiloride-sensitive currents approximately 6-fold when coexpressed in *Xenopus* oocytes. Aprotinin treatment of the $mpkCCD_{c14}$ cell line to inhibit endogenous serine protease activity again reduced amiloride-sensitive currents.

The current study was designed to identify human serine proteases expressed in the airway epithelia that may be important regulators of ENaC activity. We used RT-PCR and cDNA library screening techniques to search for sequences with homology to xCAP1 that are expressed by human tracheobronchial airway epithelial cells. Two separate clones were obtained by these methods, which were identical to previously cloned serine proteases, prostasin and TMPRSS2. The expression pattern in the lung and functional effects on ENaC in *Xenopus* oocytes were also tested. Finally, the importance of serine protease activity for ENaC regulation in airway tissues was examined using primary cultures of nasal airway epithelial cells. Based on these data, we propose that prostasin is the human homologue of xCAP1 and is involved in regulation of ENaC activity in the lung.

EXPERIMENTAL PROCEDURES

Tissue Acquisition—Human nasal tissues were obtained from patients (mean age 42.8 years; range 19–58 years) undergoing rhinologic procedures (*e.g.* turbinectomy). Upper and lower airway sections were obtained from excess donor lung tissue during lung transplantation procedures. All human tissue protocols were reviewed and approved by the University of North Carolina Committee for the Protection of the Rights of Human Subjects.

RNA Isolation, cDNA Library Preparation, PCR, and Library Screening—For RNA isolation and cDNA library preparation, trachea and third to sixth generation airways were dissected and incised longitudinally. Guanidine thiocyanate was applied directly to the surface epithelium for 1–2 min, and the epithelial digest was removed by scraping the surface with a glass slide. RNA was isolated by CsCl centrifugation, and the integrity was confirmed by gel electrophoresis. Microscopic examination of the tissue sections established that the superficial epithelium was effectively removed from the underlying structure. A portion of the isolated RNA was reverse transcribed, and a cDNA library was constructed using the ZAP-cDNA Synthesis Kit (Stratagene) as per the manufacturer's instructions.

Four degenerate primers were designed, based on the amino acid sequence of xCAP1 (outer 5′, primer A (5′-GACTATGGCGCGCCGGNAA(A/G)TT(T/C)CCNTGGCA(A/G)GT-3′); outer 3′, primer B (5′-GACTATTTAATTAATANACNCCNGGNC(G/T)(A/G)TTNGG-3′); nested 5′, primer C (5′-GACTATGGCGCGCCACNGCNGCNCA(T/C)TG(T/C)TT(T/C)CC-3′); nested 3′, primer D (5′-GACTATTTAATTAACCN(G/C)(A/T)(A/G)TCNCC(T/C)TG(A/G)CANGC-3′)). After 40 cycles of RT-PCR on human tracheobronchial RNA with primer combinations A-B, A-D, C-B, and C-D, the PCR products were reamplified with the appropriate nested primer(s) for another 40 cycles of PCR, separated on a 1.2% agarose gel, excised, purified, and ligated into the pCR2.1 vector with the TA cloning kit (Invitrogen). Transformants were screened for inserts following *Eco*RI digestion, and positive clones were sequenced by chain termination automated sequencing.

A search for other relevant serine proteases was performed by screening a human cDNA tracheobronchial epithelial library under low stringency conditions with the 654-bp PCR product amplified using the A-B primers. Library filters were incubated in prehybridization solution (50% formamide; 1 h) and then exposed to [α^{32}P]dCTP-labeled probe (Random Primed labeling kit; Roche Molecular Biochemicals) in hybridization solution (30% formamide; 37 °C) overnight. Filters were then sequentially washed with 2× SSC, 0.1% SDS (2 × 15 min) and with 0.5× SSC, 0.1% SDS (2 × 15 min) at 37 °C before exposing to radiographic film overnight at −80 °C. Positive clones were released by *in vivo* phage excision and identified by chain termination automated sequencing. Further library screening with full-length probes encoding xCAP1, prostasin, and TMPRSS2 was also performed under the identical conditions described above.

In Situ Hybridization—For *in situ* hybridization studies, tissues were rapidly dissected from several regions (nasal, tracheal, and distal lung) and snap frozen in OCT embedding compound, typically within 30 min of tissue resection. Distal lung sections were frozen on dry ice following inflation with a 1:1 solution of OCT embedding compound and phosphate-buffered saline. Thin sections (8 μm) were cut by a cryothome and mounted on glass slides. Tissue sections were fixed in 4% paraformaldehyde for 60 min, dehydrated through a graded alcohol series, and stored desiccated at −20 °C until analyzed.

In situ hybridization was performed as previously described (18). Fragments of the prostasin (bp 584–1039) and TMPRSS2 (bp 801–1205) coding regions were subcloned into the pBluescript KS+ vector, and ^{35}S-UTP-labeled sense and antisense riboprobes were synthesized by *in vitro* transcription with the appropriate RNA polymerase (Promega). Cryosections were air-dried, digested with proteinase K (10 μg/ml; 30 min at 30 °C), and hybridized with the appropriate ^{35}S-UTP-labeled riboprobe (~5 × 10^6 cpm in 50 μl). After washing and RNase treatment, tissue sections were coated with photographic emulsion (Kodak NTB-2) and exposed for 5–14 days. Sections were then developed, counterstained with hematoxylin and eosin, and examined with a Nikon microphot SA microscope connected to a 3CC-Chilled Camera (Sony).

Functional Analysis in the Oocyte Expression System—cRNAs encoding xCAP1, xENaC, and $rENaC^{FLAG}$ subunits; β_2-adrenergic receptor (cDNAs kindly provided by B. C. Rossier; Lausanne, Switzerland), CFTR, prostasin, and TMPRSS2 were synthesized using SP6 or T7 RNA polymerase, analyzed for integrity with gel electrophoresis, and quantitated by spectrophotometry. Healthy stage V-VI oocytes were harvested from *Xenopus laevis* (*Xenopus*-1; Dexter, Michigan), defolliculated in collagenase type IA (1 mg/ml; Sigma) in calcium-free modified Barth's solution, and maintained at 18 °C in modified Barth's solution (85 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 10 mM Hepes-NaOH (pH 7.2), 10 mg/liter penicillin, and 5 mg/liter streptomycin). Twenty-four hours after collagenase treatment, oocytes were injected with 100 nl of the designated cRNA solution. To test the effects of prostasin and TMRPSS2 on ENaC, a solution containing equal amounts of αβγ ENaC subunits (0.1–0.3 ng/subunit/oocyte) and 0.75–2 ng/oocyte of the designated serine protease cRNA was injected. To test the effect of TMPRSS2 on CFTR, oocytes were injected with 10 ng of CFTR cRNA, 1 ng of β_2-adrenergic receptor cRNA, and 2 ng of TMPRSS2 cRNA.

Two-electrode voltage clamp experiments were performed 18–24 h after the injection of cRNAs. Injected oocytes were perfused in frog Ringer solution (containing 120 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM Hepes, pH 7.2), and currents were measured at a holding potential of −100 mV. The difference in current measured in the presence and absence of amiloride (10 μM) defined ENaC currents. Substitution of NaCl with 120 mM LiCl or KCl allowed the determination of ion selectivity through ENaC in oocytes expressing ENaC with or without prostasin. The effect of aprotinin (100 μg/ml) or protease inihibitor mixture (containing 4-(2-aminoethyl)benzenesulfonyl fluoride (100 μM), pepstatin A (1.5 μM), *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (1.4 μM), bestatin (4 μM), leupeptin (2.2 μM), and aprotinin (80 nM)) on protease-mediated ENaC regulation were tested by adding these reagents either immediately after cRNA injection or 5 h before voltage clamping oocytes. CFTR currents were measured in response to the addition of isoproterenol (1 μM) to oocytes expressing both CFTR and the β_2-adrenergic receptor.

Western blot analysis was used to examine prostasin protein expression in primary cultured airway epithelia and to test for the expression of cRNAs injected in oocytes. Epithelial cell proteins were harvested from 12-mm T-col inserts into 400 μl of radioimmune precipitation buffer (containing 150 mM NaCl, 50 mM Tris-Cl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.5% Triton X-100, 1% SDS, 20 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin A) using a cell scraper and transferred to Eppendorf tubes. After briefly vortexing, cells were incubated on ice for 30 min and then centrifuged for 5 min at 14,000 × *g* to removed insoluble material. Soluble cell supernatant was collected and electrophoresed as described below.

Protein extracted from oocytes was isolated by serial passage through 22- and 27-gauge needles in homogenization buffer (containing 77 mM NaCl, 10 mM Hepes, 1 mM $MgCl_2$, pH 7.9, 20 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin A) on ice. Centrifugation at 1000 × *g* (4 °C) for 5 min separated yolk and pigment granules from the remainder of the cell lysate. Further centrifugation at 14,000 × *g* for 20 min (4 °C) pelleted a microsomal fraction. Protein

concentrations were measured using the Bradford method (Bio-Rad), and 25–50 μg of cell lysate or microsomal fraction was dissolved in sample buffer and heated to 70 °C for 10 min prior to loading onto a 4–15% gradient acrylamide gel. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane, blocked with 5% nonfat dry milk, and incubated with primary antibody (either m2-FLAG (1:1000; Sigma), a polyclonal antibody raised against the amino terminus of α-rENaC (αN, 1:300; kindly provided by D. Rotin), or a polyclonal antibody raised against prostasin) for 1 h at room temperature. Exposure to secondary antibody (anti-mouse or -rabbit conjugated to horseradish peroxidase, 1:5000–10,000; Amersham Biosciences, Inc.) for 1 h at room temperature followed by detection with ECL reagents (Amersham Biosciences) was used to reveal immunoreactive proteins.

Ussing Chamber Experiments—Human nasal epithelial cells harvested from surgical tissue specimens were seeded at a density of $0.25 \times 10^6/cm^2$ on porous Snapwell®-permeable supports (1.13-cm^2 surface area; Costar) coated with human placental collagen (50 μg/cm^2; Sigma type VI) as previously described (19). Cell monolayers were grown at an air-liquid interface in F-12/Dulbecco's modified Eagle's medium (1:1) with 2% Ultraser-G. Cells were maintained in a humidified, 5% CO_2, 37 °C incubator, and the culture medium was changed every other day. Transepithelial resistance (R_t) and potential difference were monitored daily, and cultures were used after ≥7 days of air-liquid interface culture conditions.

Primary nasal epithelial air-liquid interface cultures exhibiting $R_t >$ 150 Ω·cm^2 were mounted in modified Ussing chambers (Physiologic Instruments). The epithelium was bathed on both sides with warmed (37 °C) Krebs-bicarbonate Ringer solution (containing 140 mM Na^+, 120 mM Cl^-, 5.2 mM K^+, 1.2 mM Ca^{2+}, 1.2 mM Mg^{2+}, 2.4 mM HPO_4^{2+}, 0.4 mM $H_2PO_4^-$, 25 mM HCO_3^-, and 5 mM glucose), circulated by gas lift with 95% O_2, 5% CO_2. The epithelial culture was voltage-clamped (Physiological Instruments), and the short circuit current (I_{sc}) was measured and continuously recorded (Acquire version 1.0; Dataq Instruments). After establishing a stable I_{sc}, aprotinin (20 μg/ml, ~5 trypsin inhibitory units/mg; Sigma) was added to the apical bath. After a 75-min incubation, a brief exposure to amiloride (100 μM; Sigma) was used to measure the ENaC-mediated current. A 3-fold excess of trypsin (200 μg/ml, ~10,000 *N*-benzoyl-L-arginine ethyl ester units/mg; Sigma) on the basis of activity (1 trypsin unit = 9000 *N*-benzoyl-L-arginine ethyl ester units) was then added, followed by a second exposure to amiloride.

Statistics—Results are expressed as the means ± S.E. Amiloride-sensitive currents in oocytes coexpressing ENaC with serine proteases were normalized to currents measured in ENaC-expressing oocytes studied from the same animal on the same day. Significance was determined using an unpaired Student's *t* test or analysis of variance for the comparison of multiple sample groups.

RESULTS

Identification and Isolation of Two Human Serine Protease cDNAs Expressed in Airways—Expressed genes with sequence homology to xCAP1 were identified by RT-PCR of airway epithelial RNA and by screening an airway cDNA expression library. Using degenerate primers based upon the xCAP1 amino acid sequence, RT-PCR generated a ~600-bp product that was sequenced and found to be identical to a portion of the human prostasin gene open reading frame. This PCR product and the full-length prostasin and xCAP1 cDNA sequences were then used to screen an airway epithelial expression library under low stringency conditions in an attempt to identify other sequences with significant homology. Using this approach, only one other homologous clone was identified. This clone was sequenced and found to be identical to another previously cloned serine protease, TMPRSS2. Full-length prostasin and TMPRSS2 clones were then generated by PCR using the TA cloning kit (Invitrogen), and sequences were verified by chain termination automated sequencing. Search of the GenBank™ data bases revealed that prostasin is the nearest human homologue to xCAP1 and mCAP1 (41 and 76% amino acid identity, respectively). TMPRSS2, on the other hand, is significantly less homologous to the mouse and *Xenopus* CAP1 sequences, having only about ~16% identity (Fig. 1).

Expression of Prostasin and TMPRSS2 in Human Lung—To evaluate the pattern of prostasin and TMPRSS2 expression in the lung, we performed *in situ* hybridization on tissue specimens obtained from the nose, upper airway, and distal lung (Figs. 2 and 3). In these studies, we found strong expression of both prostasin and TMPRSS2 in superficial epithelial cells lining the nose, trachea, and distal airways. Although difficult to assess because of the fine structural architecture, both prostasin and TMPRSS2 appeared to be expressed at the alveolar level, most notably at alveolar junctions in a "cornering pattern" characteristic of type II pneumocytes. In addition to robust surface epithelial expression, message encoding prostasin and TMPRSS2 was also pronounced in submucosal glands associated with nasal, tracheal, and bronchial tissues. Importantly, neither serine protease was observed in nonepithelial tissues.

Functional Analysis of Prostasin and TMPRSS2 in Xenopus Oocytes—To determine whether prostasin or TMPRSS2 could regulate ENaC currents, we performed cRNA coexpression studies in *Xenopus* oocytes. Current measured via two-electrode voltage clamping 24 h after cRNA injection in the presence and absence of amiloride defined ENaC-mediated currents. Importantly, amiloride-sensitive currents were not observed in control oocytes injected only with water, prostasin, or TMPRSS2 (data not shown). Coexpression of prostasin cRNA with either *Xenopus* or rat ENaC cRNA, however, led to an ~80% increase in rENaC-mediated currents ($p < 0.005$; $n \geq$ 73 oocytes/group from 10 frogs) and a ~60% increase in xENaC-mediated currents ($p = 0.02$; $n \geq 28$ oocytes/group from three frogs) (Fig. 4*A*). We also measured the ability of xCAP1 to activate ENaC in parallel groups of oocytes and observed a similar magnitude of channel activation in xENaC-expressing oocytes (p = NS; $n = 24$ oocytes from three frogs) (Fig. 4*A*). Interestingly, xCAP1 caused a significantly greater activation of amiloride-sensitive currents in rENaC-expressing oocytes than did prostasin ($p < 0.01$; $n = 46$ oocytes from seven frogs) (Fig. 4*A*). We confirmed that the activation of ENaC by prostasin required extracellular serine protease activity by incubating oocytes in aprotinin (100 μg/ml) for 5 h prior to measurement of amiloride-sensitive currents. This maneuver completely prevented ENaC activation by prostasin ($p < 0.001$; $n \geq 7$ oocytes/group) (Fig. 4*B*). Aprotinin treatment also reduced ENaC currents in oocytes expressing ENaC alone ($p <$ 0.005; $n \geq 7$ oocytes/group)(Fig. 4*B*), suggesting that an endogenous protease with the ability to activate ENaC may also be expressed in oocytes. The addition of trypsin (200 μg/ml) after aprotinin treatment increased ENaC currents ~5-fold within 3 min in both groups ($p < 0.001$; $n \geq 7$ oocytes/group) (Fig. 4*B*), demonstrating that ENaC channels remained sensitive to protease activation after aprotinin treatment.

We tested whether coexpression of prostasin with rENaC changed the electrophysical properties of the channel by determining the amiloride-sensitive current carried by Na^+, Li^+, or K^+ in the presence and absence of prostasin. No significant amiloride-sensitive current was measured at −100 mV when K^+ was the only monovalent cation in the extracellular bath either in the presence or absence of prostasin. In agreement with data from native tissues, Li^+ was conducted to a greater extent than Na^+ in both groups of oocytes. However, we observed a trend toward a lower Li^+/Na^+ conductivity ratio in oocytes coexpressing prostasin with ENaC (1.39 ± 0.02 *versus* 1.60 ± 0.10; $p = 0.13$, $n = 5$ per group).

In contrast to prostasin, coexpression of TMPRSS2 with ENaC led to a marked reduction in amiloride-sensitive currents (Fig. 5*A*). The effect was directly proportional to the amount of TMPRSS2 cRNA that was injected in each oocyte, and obliteration of ENaC currents was achieved with relatively small amounts of injected TMPRSS2 cRNA (>2 ng/oocyte) (Fig.

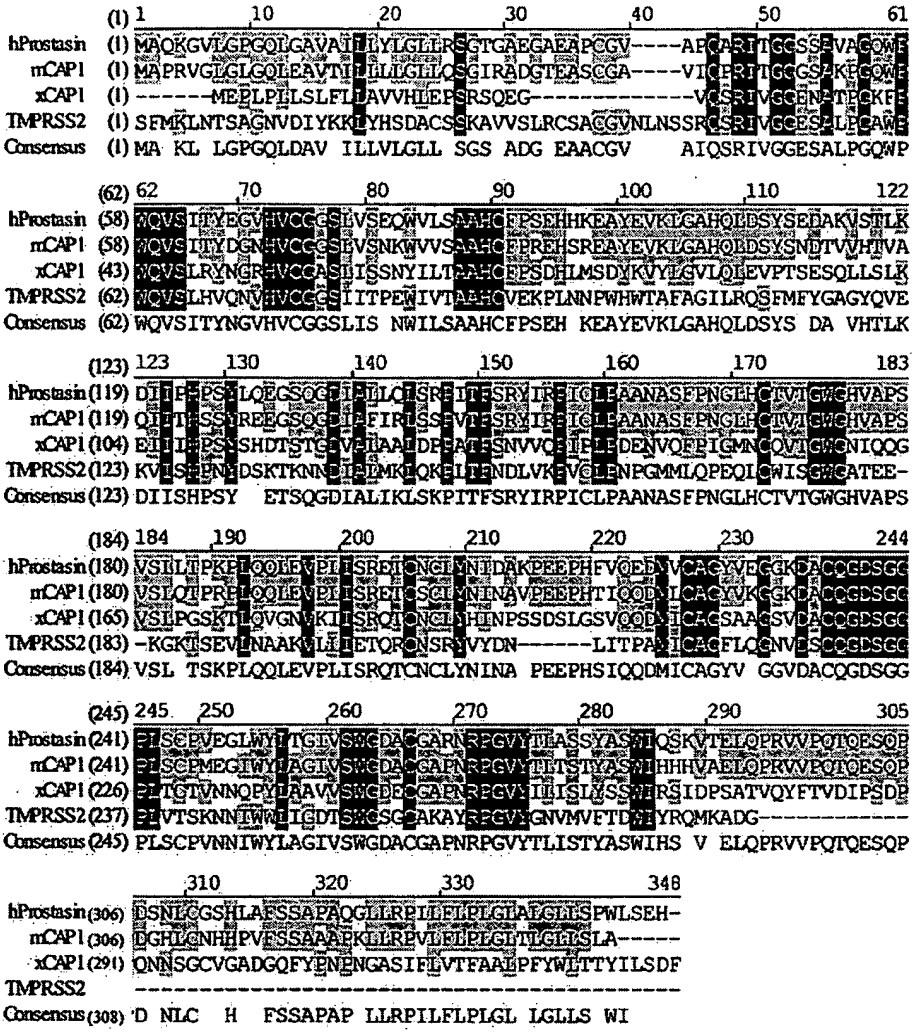

FIG. 1. **Amino acid alignment of relevant serine proteases.** Sequences of human prostasin, mouse CAP1, *Xenopus* CAP1, and human TMPRSS2 are aligned with identical residues in ***boldface type*** and similar residues *shaded*. The nonhomologous amino terminus of TMPRSS2 (residues 1–207) is not shown.

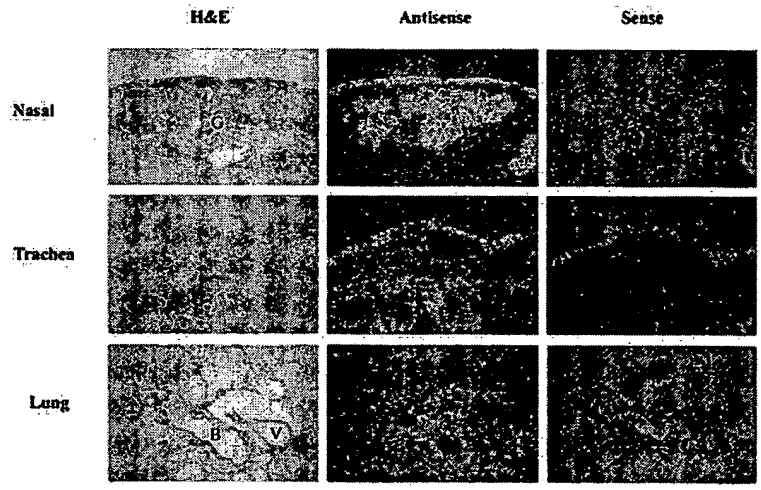

FIG. 2. ***In situ* hybridization of prostasin in nasal, tracheal, and distal lung tissues.** The bright field image (*H&E*) of each frozen section (*left*) and dark-field images of sections exposed to antisense (*center*) and sense (*right*) mRNA probes are shown. Submucosal glands (*G*), bronchioles (*B*), and blood vessels (*V*) are labeled accordingly.

5*B*). To test whether this affect was due to a generalized inhibition of protein translation, TMPRSS2 was coexpressed with CFTR and the β_2-adrenergic receptor (Fig. 5*C*). In these experiments, CFTR currents stimulated by isoproterenol were not significantly reduced compared with CFTR currents measured in the absence of TMPRSS2.

Reduced ENaC currents may also be the result of a specific effect on ENaC protein levels. We prepared yolk-depleted ly-

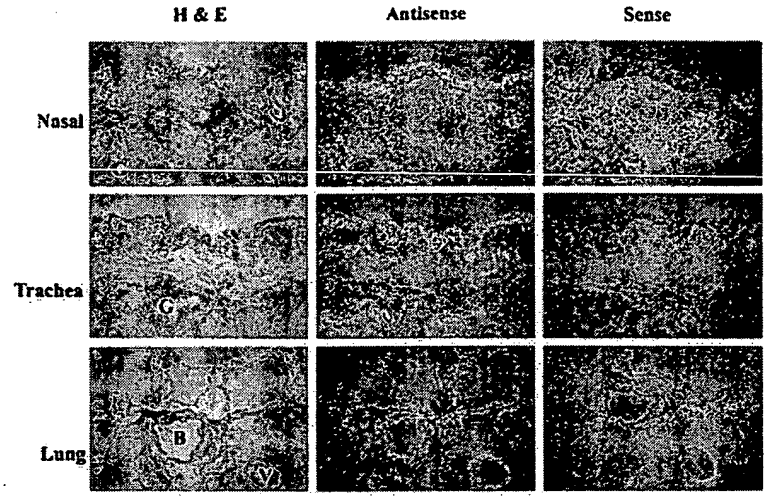

FIG. 3. **In situ hybridization of TMPRSS2 in nasal, tracheal, and distal lung tissues.** The bright field image (*H&E*) of each frozen section (*left*) and dark-field images of sections exposed to antisense (*center*) and sense (*right*) mRNA probes are shown. Submucosal glands (*G*), bronchioles (*B*), and blood vessels (*V*) are labeled accordingly.

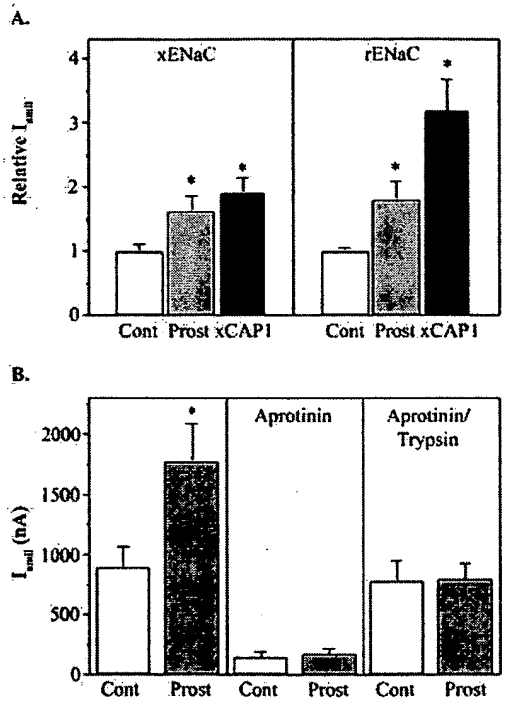

FIG. 4. **Functional analysis of prostasin in *Xenopus* oocytes.** *A*, oocytes were injected with cRNA encoding *Xenopus* ($n \geq 24$ oocytes from three animals per condition) or rat ($n \geq 46$ oocytes from at least seven animals per condition) ENaC subunits (0.3 ng/subunit) in the absence (*open bars*) or presence of human prostasin (2 ng) (*gray bars*) or xCAP1 (2 ng) (*black bars*), and amiloride-sensitive currents were measured. *B*, oocytes were injected with cRNA encoding rENaC in the absence (*open bars*) or presence of human prostasin (*gray bars*). Amiloride-sensitive currents were measured after no treatment (*left panel*; $n \geq 7$ oocytes/group) and 5 h after exposing oocytes to aprotinin (100 μg/ml) (*center panel*; $n \geq 7$ oocytes/group). The subsequent effect of trypsin (200 μg/ml) on amiloride-sensitive currents in aprotinin-pretreated eggs was also tested (*right panel*; $n \geq 7$ oocytes/group). *, $p < 0.05$ *versus* control.

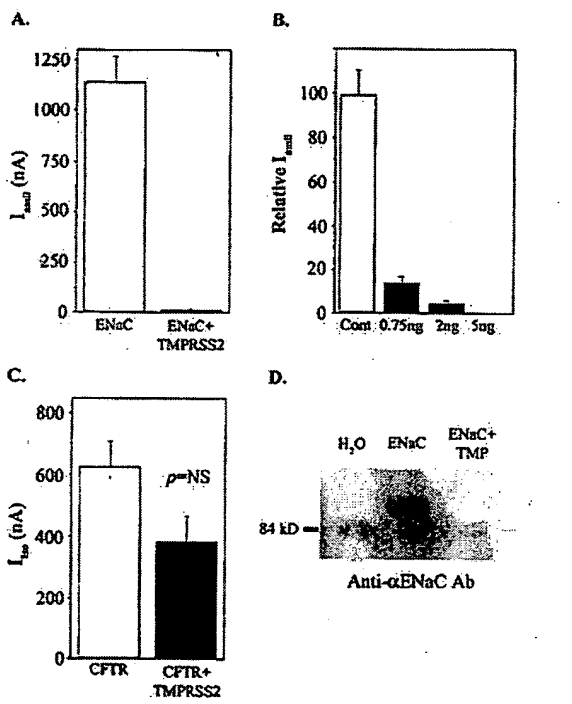

FIG. 5. **Functional analysis of TMPRSS2 in *Xenopus* oocytes.** *A*, oocytes were injected with cRNA encoding rENaC subunits (1 ng cRNA) in the absence (*open bars*; $n = 65$) or presence (*black bars*; $n = 14$) of TMPRSS2 (2 ng of cRNA), and amiloride-sensitive currents were measured. *B*, dose response of TMPRSS2 on ENaC-mediated currents (*, $p < 0.001$ between groups by analysis of variance; $n \geq 14$ oocytes/group). *C*, CFTR (10 ng cRNA) and the β_2-adrenergic receptor (1 ng cRNA) were coinjected in the presence or absence of TMPRSS2 (2 ng) (p = NS; n = 7 oocytes/group). *D*, Western analysis, using polyclonal antibody directed against the α subunit of rat ENaC.

sates and microsomal fractions from oocytes expressing rENaC with or without TMPRSS2 and measured ENaC protein expression by Western blot analysis. In these experiments, expression of TMPRSS2 resulted in a virtually complete loss of ENaC protein (Fig. 5*D*). In contrast to the observations with prostasin, the inhibition of ENaC currents by TMPRSS2 was not prevented by the addition of aprotinin (200 μg/ml) or a mixture of protease inhibitors added to the oocyte bathing media immediately after cRNA injection (data not shown), suggesting that channel inactivation/degradation did not occur after ENaC and TMPRSS2 had reached the plasma membrane.

Effect of Aprotinin and Trypsin on Human Airway Tissues—Well differentiated primary nasal airway epithelial cultures were mounted in modified Ussing chambers to examine ENaC regulation by endogenous serine proteases (Fig. 6, *A* and *B*). The addition of aprotinin (10 μM) to the apical bath resulted in a marked inhibition of the I_{sc} over 75 min, compared with vehicle controls ($p = 0.01$). The subsequent addition of trypsin led to a rapid increase in I_{sc} in tissues that had been pretreated with aprotinin ($p < 0.001$). Importantly, nearly the entire I_{sc}

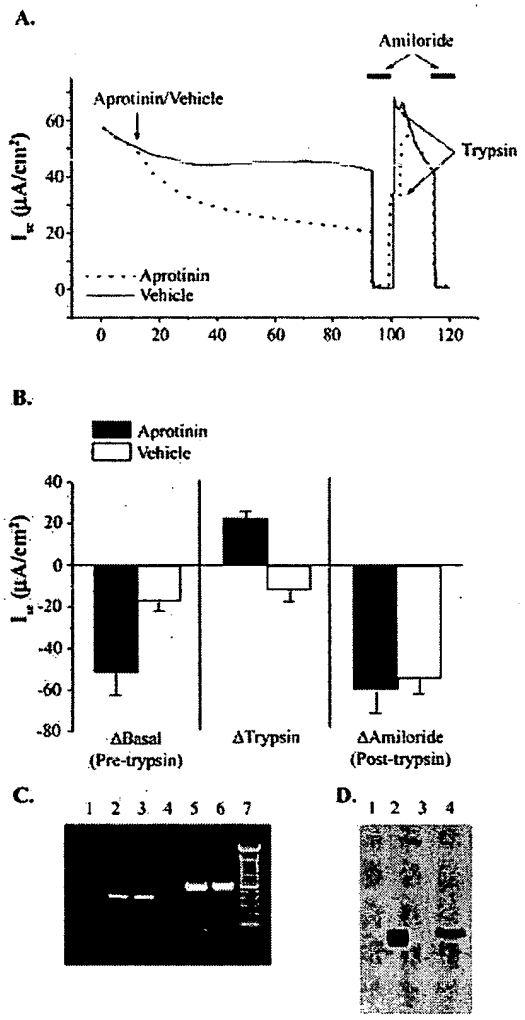

FIG. 6. **Effect of aprotinin and trypsin on short circuit current (I_{sc}) in primary nasal epithelial cells.** Cells were mounted in modified Ussing chambers and exposed to aprotinin (20 μg/ml) or vehicle for 75 min; all cells were then exposed to trypsin (200 μg/ml). *A*, representative tracing of the described experiment. *B*, summary data from three tissue donors (n = 9 tissues), expressed as the change in I_{sc} between the addition of aprotinin (*black bars*) or vehicle (*open bars*) and 75 min later; the change in I_{sc} in response to trypsin, measured 5 min after its addition in aprotinin-treated (*black bars*) and -untreated (*open bars*) tissues; and the change in I_{sc} after amiloride, added after trypsin. *C*, expression of prostasin and TMPRSS2 in primary nasal epithelial cell cultures was confirmed using RT-PCR. Prostasin primers were used in *lanes 1–3*; TMPRSS2 primers were used in *lanes 4–6*. *Lanes 1* and *4* were reactions performed in the absence of reverse transcriptase; *lanes 2* and *5* were performed cDNA-positive controls; *lanes 3* and *6* were performed with mRNA isolated from primary nasal epithelial cell cultures. *D*, prostasin protein expression was confirmed by Western blot analysis. *Lanes 1* and *2* contain oocyte microsomal fractions made from uninjected (*lane 1*) and prostasin-expressing oocytes (*lane 2*) and are included as controls. *Lane 3* is empty, and *lane 4* was loaded with primary airway epithelial cell lysate (20 μg).

was amiloride-sensitive. These data suggest that endogenous serine proteases had an activating influence on ENaC in primary cultured cells. We therefore determined whether prostasin and TMPRSS2 mRNAs were expressed in this cell culture system using RT-PCR (Fig. 6*C*). As shown, both serine proteases were expressed in identical tissues to those used for Ussing chamber experiments. Prostasin protein was also readily detected in primary culture airway epithelia (Fig. 6*D*), although antibodies raised against TMPRSS2 proved not to be useful due to poor specificity.

DISCUSSION

The regulation of ENaC activity is tightly linked to ASL volume and mucociliary clearance and therefore is a key determinant of lung health. ENaC regulation appears to be tissue-specific, and in the lung it may rely upon regulators of ENaC that are responsive to local stimuli. The discovery of an extracellular serine protease that is expressed on the lumen of *Xenopus* (16) and mouse (17) renal epithelia and can regulate ENaC activity raised the possibility that a similar mechanism of ENaC regulation might exist in the human lung.

In the present study, we sought to determine whether a channel-activating serine protease was expressed in human airway epithelia. Using RT-PCR and cDNA library screening techniques, we identified two candidate serine proteases, prostasin and TMPRSS2, that were expressed in human tracheobronchial epithelial cells. Sequence analysis of prostasin and TMPRSS2 showed that both belong to the trypsin family of serine proteases (peptidase family S1). Comparison of prostasin and TMPRSS2 with xCAP1 and mCAP1 reveals considerable similarity between prostasin and the CAP1 genes and significantly less similarity between TMPRSS2 and either of the other sequences.

Interestingly, both prostasin and TMPRSS2 are predicted to be plasma membrane proteins, with protease catalytic domains located in the extracellular compartment (20, 21). The protein structure of prostasin predicts an amino-terminal signal peptide (residues 1–29), an extracellular trypsin-like protease domain, and a COOH-terminal transmembrane domain (residues 320–340). Yu *et al.* (20) further deduced that prostasin purified from seminal fluid was a heterodimer, formed by cleavage of a precursor prostasin polypeptide into a 12-amino acid light chain (residues 33–44) and 299-amino acid heavy chain (residues 45–322). Secretion into the prostatic lumen, therefore, required cleavage between the heavy chain domain and the COOH-terminal transmembrane domain. Further work has shown that prostasin, like xCAP1, is a glycosylphosphatidylinositol-anchored protein that may either be secreted or membrane-bound (22). Consistent with our *in situ* hybridization and library screening data, Yu *et al.* (20) detected prostasin mRNA in the lung by Northern analysis as well as in other epithelia-lined organs (prostate, liver, salivary gland, kidney, pancreas, and colon). Although very high protein levels have been detected in the prostate (23), its function in this and other tissues remains unknown.

In contrast to CAP1 and prostasin, the TMPRSS2 sequence predicts a type II transmembrane protein without a recognizable signal peptide, an amino-terminal cytoplasmic domain (residues 1–84), a transmembrane domain (residues 85–105), and a complex extracellular structure (residues 106–492) that not only includes a serine protease domain but also low density lipoprotein receptor A and scavenger receptor cysteine-rich domains (21). Low density lipoprotein receptor A and scavenger receptor cysteine-rich motifs are thought to mediate low density lipid/Ca^{2+} binding and the binding of extracellular molecules to the cell surface, respectively. Because TMPRSS2 may be up-regulated in the basal cell layer of malignant prostate cancers, a role in carcinogenesis has been proposed, although its function in normal cellular physiology is not yet known (24). Once again, consistent with our data, other investigators have detected the expression of TMPRSS2 in the lung at the mRNA level (21, 24).

Functional testing of prostasin and TMPRSS2 in *Xenopus* oocytes revealed opposite effects on ENaC-mediated Na^+ currents. Prostasin, like xCAP1, activated amiloride-sensitive Na^+ currents in oocytes. Previous studies have shown that xCAP1 and exogenous trypsin activate ENaC without an asso-

ciated increase in the number of surface channels or change in single channel conductance, thereby implicating a marked increase in channel open probability (16, 17, 25). The mechanism underlying this change in channel activity is unknown, but it could reflect a direct effect of the protease on ENaC or may involve intermediary proteins. In this latter scenario, one may envision either the proteolytic destruction/alteration of an ENaC-inhibiting molecule or the proteolytic activation of a separate channel-activating preprotein. We did observe a small change in the relative conductance of lithium and sodium through ENaC channels that were coexpressed prostasin, possibly suggesting a direct effect on the channel itself. Interestingly, a similar change in ion selectivity was previously observed after treating ENaC-expressing oocytes with trypsin (25). Further studies are necessary to investigate these potential mechanisms.

In contrast, TMPRSS2 expression abolished ENaC-mediated amiloride-sensitive Na^+ currents in oocytes. Furthermore, TMPRSS2 expression also markedly reduced cellular ENaC protein levels. Given the absence of an effect of TMPRSS2 on CFTR and β_2-adrenergic receptor expression, oocyte health and the capacity to translate cRNA into protein were intact in oocytes expressing TMPRSS2. We also ruled out the simple explanation that oocytes preferentially express CFTR > TMPRSS2 > ENaC through CFTR/ENaC coexpression studies. In these experiments, CFTR coexpression did not reduce ENaC-mediated currents (prior to CFTR activation with cAMP), nor did it reduce total ENaC protein levels (data not shown). Channel proteolysis by TMPRSS2 may instead be responsible for the observed decrease in ENaC activity. The site of proteolysis is likely to be in the intracellular compartment, because the addition of protease inhibitors to the bathing media did not alter the observed inhibition of ENaC currents. Certainly, the major question to be raised by these observations is whether TMPRSS2 proteolysis of ENaC is physiologically relevant. Because airway epithelia have ENaC currents that are stimulated by endogenous serine protease activity (see below), it seems likely that ENaC inhibition by TMPRSS2 is not a dominant regulatory mechanism. These data do suggest that not all trypsin-like serine proteases are able to stimulate ENaC and/or that cellular compartmentalization of the channel and protease may contribute to specificity.

Using well differentiated airway epithelia, aprotinin and trypsin were used as probes of the endogenous extracellular serine protease activity in human airway epithelia. Under the conditions used in these experiments, sodium currents through ENaC are substantial and represent the dominant basal ion transport activity (Fig. 6, *A* and *B*). Treatment with apical aprotinin led to a gradual but clear decline in the amiloride-sensitive I_{sc} over 75 min. The subsequent addition of excess trypsin to the apical chamber caused an immediate increase in I_{sc}, which was entirely amiloride-sensitive. This trypsin response was only observed in aprotinin-pretreated tissues. These data, therefore, demonstrate that an endogenous serine protease is present, activates ENaC, and is fully effective at endogenously expressed levels, as evidenced by the observation that trypsin does not activate ENaC in the absence of aprotinin pretreatment (Fig. 6*B*). Based upon its homology to CAP1, airway expression, and these functional studies, we speculate that prostasin, and not TMPRSS2, is likely to be a serine protease relevant to ENaC regulation in airway epithelia. It is possible, however, that other serine proteases, not detected by our library screening experiments, may also contribute to regulation of ENaC in the human airway. In addition, while ENaC is thought to be the dominant amiloride-sensitive pathway in airway epithelia, we have not ruled out a serine protease effect on other amiloride-sensitive, electrogenic pathways (*e.g.* cyclic nucleotide gated channels (26)), which could contribute to our experimental results.

With the identification of this novel regulatory pathway come additional questions regarding the regulation of prostasin activity in airways. Because airway surfaces are in direct communication with inspired air, ASL is subject to fluctuations in ionic strength and pH. One may envision that the catalytic activity of prostasin and ultimately the activity of ENaC may be regulated by changes such as these in the local milieu. Another possibility is that the total amount of prostasin available to regulate ENaC might also be regulated acutely. As shown by Yu *et al.*, prostasin is not only cell-associated but is also secreted by prostatic epithelial cells (23). Because we have shown that airway submucosal glands also contain high levels of prostasin message, it is possible that signals responsible for gland secretion in airways might also lead to prostasin secretion in airways populated by submucosal glands (*i.e.* proximal, cartilaginous airways). In this manner, both surface-associated and secreted prostasin may contribute to ENaC regulation. Finally, because the stoichiometry of ENaC channel subunits may vary in different lung regions (18, 27), it is also possible that the impact of serine proteases on sodium absorption may vary in different lung regions.

Further regulation of prostasin may also occur via endogenous serine protease inhibitors in airways. In fact, in most tissues that express a serine protease, a protease inhibitor is also expressed to prevent uncontrolled tissue proteolysis. Of note, a serine protease inhibitor termed placental bikunin was recently cloned based on homology with aprotinin, is expressed in lung, and has the potential to be either a membrane-associated or secreted protein (28, 29). Although we did not observe further activation of ENaC by exogenous trypsin without aprotinin pretreatment in Ussing chambers (suggesting the absence of an endogenous protease inhibitor), failure to observe this interaction may reflect massive dilution (4 ml/cm^2) of a soluble inhibitor in this experimental system, which is not representative of the protease/inhibitor balance present under thin liquid ASL conditions. Whether or not bikunin or another relevant serine protease inhibitor is present on apical membranes or in the airway lumen remains to be determined.

In summary, our data show that ENaC expressed in airway epithelial cells is regulated by serine protease activity located in the extracellular compartment. We propose that prostasin is a relevant protease involved in this process and appears to be the human homologue of the CAP1 gene. It is possible that prostasin may be able to mediate ENaC regulation in response to changes in the channel's immediate microenvironment. Future studies will focus on the mechanisms underlying channel activation by prostasin and the larger issue of ENaC regulation in airways.

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